Promising role of toll-like receptor 8 agonist in concert with prostratin for activation of silent HIV

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Abstract: The persistence of latently HIV-infected cells in patients under combined anti-retroviral treatment (cART) remains the major hurdle for HIV eradication. Thus far, individual compounds have not been sufficiently potent to reactivate latent virus and guarantee its elimination in vivo Thus, we hypothesized that transcriptional enhancers, in concert with compounds triggering the innate immune system, are more efficient in reversing latency by creating a Th1 supportive milieu that acts against latently HIV-infected cells at various levels. To test our hypothesis, we screened six compounds on a co-culture of latently infected cells (J-lat) and monocyte-derived dendritic cells (MDDCs). The PKC agonist, Prostratin, with a TLR8 agonist, resulted in greater reversion of HIV latency than any single compound. This combinatorial approach led to a drastic phenotypic and functional maturation of the MDDCs. TNF and cell-cell interactions were crucial for the greater reversion observed. Similarly, we found a greater potency of the combination of Prostratin/TLR8 agonist in reversing HIV latency when applying it to primary cells of HIV-infected patients. Thus, we demonstrated here the synergistic interplay between TLR8-matured MDDCs and compounds acting directly on latently HIV-infected cells, targeting different mechanisms of latency, by triggering various signaling pathways. Moreover, TLR8 triggering may reverse exhaustion of HIV-specific cytotoxic T lymphocytes that might be essential for killing or constraining the latently infected cells. IMPORTANCE Curing HIV is the Holy Grail. The so-called "shock and kill strategy" relies on drug-mediated reversion of HIV latency and the subsequent death of those cells under combined anti-retroviral treatment. So far, no compound achieves efficient reversal of latency nor eliminates this latent reservoir. The compounds may not target all of the latency mechanisms in all latently infected cells. Moreover, HIV-associated exhaustion of the immune system hinders the efficient elimination of the reactivated cells. In this study, we demonstrated synergistic latency reversion by combining agonists for protein kinase C and toll-like receptor 8 in a co-culture of latently infected cells with myeloid dendritic cells. The drug Prostratin stimulates directly the transcriptional machinery of latently infected cells and the TLR8 agonist acts indirectly by maturing dendritic cells. These findings highlight the importance of the immune system and its activation, in combination with direct acting compounds, to reverse latency.

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Promising Role of Toll-Like Receptor 8 Agonist in Concert with Prostratin for Activation of Silent HIV

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ABSTRACT

The persistence of latently HIV-infected cells in patients under combined anti-retroviral treatment (cART) remains the major hurdle for HIV eradication. Thus far, individual compounds have not been sufficiently potent to reactivate latent virus and guarantee its elimination *in vivo*. Thus, we hypothesized that transcriptional enhancers, in concert with compounds triggering the innate immune system, are more efficient in reversing latency by creating a Th1 supportive milieu that acts against latently HIV-infected cells at various levels. To test our hypothesis, we screened six compounds on a co-culture of latently infected cells (J-lat) and monocyte-derived dendritic cells (MDDCs). The PKC agonist, Prostratin, with a TLR8 agonist, resulted in greater reversion of HIV latency than any single compound. This combinatorial approach led to a drastic phenotypic and functional maturation of the MDDCs. TNF and cell-cell interactions were crucial for the greater reversion observed. Similarly, we found a greater potency of the combination of Prostratin/TLR8 agonist in reversing HIV latency when applying it to primary cells of HIV-infected patients. Thus, we demonstrated here the synergistic interplay between TLR8-matured MDDCs and compounds acting directly on latently HIV-infected cells, targeting different mechanisms of latency, by triggering various signaling pathways. Moreover, TLR8 triggering may reverse exhaustion of HIV-specific cytotoxic T lymphocytes that might be essential for killing or constraining the latently infected cells.

46 **IMPORTANCE**

47 Curing HIV is the Holy Grail. The so-called “shock and kill strategy” relies on drug-
48 mediated reversion of HIV latency and the subsequent death of those cells under
49 combined anti-retroviral treatment. So far, no compound achieves efficient reversal of
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58 immune system and its activation, in combination with direct acting compounds, to
59 reverse latency.

INTRODUCTION

The HIV-1 pandemic remains a major global health threat. While combined anti-retroviral treatment (cART) effectively suppresses HIV replication, the virus rebounds when treatment is interrupted (1-5), pointing to a reservoir of latently infected cells. Indeed, a replication-competent but silent reservoir in long-lived latently HIV-infected cells is established early after HIV transmission and is not targeted by cART (6-8).

The latently infected cells are primarily resting memory CD4+ T cells. Latency is maintained by restricting access of the transcriptional machinery to the proviral DNA (9-13), and epigenetic regulation further constrains the positive Tat feedback loop (14). Moreover, key transcription factors, such as NFκB (15-17) and NFAT, as well as the transcription elongation factor PTEFb (18-22), are tightly sequestered in the resting state.

Latency may be reversed by releasing transcriptional or epigenetic blocks, decreasing the cell activation threshold (PD-1, LAG-3), or enhancing the transcriptional noise (23). The “shock and kill” strategy takes advantage of these (24-26). Reactivation of HIV production induces direct or indirect cell death that is mediated by HIV-specific cytotoxic T lymphocytes (CTL), natural killer (NK) cells (27), or antibody-dependent cellular cytotoxicity (ADCC) under cART (28, 29). This strategy should result in a sterilizing cure. However, clinical trials (e.g., using IL-2 (30-33) alone or in concert with IFN-γ (34), IL-7 (35-37) or the CD3 antibody OKT-3) have been disappointing: all the patients relapsed upon cART interruption. Moreover, in the case of OKT-3 (38), CD4+ T cell levels dropped dramatically or even an expansion of the proviral reservoir was observed for IL-7 (36).

Recent oncological clinical trials (39-41) have encouraged the *in vivo* use of protein kinase C agonists (PKCag) in humans. PKCag (i.e., Prostratin, a non-

85 tumorigenic phorbol ester (42-46), Bryostatin (47, 48) and Ingenol (49, 50)) are
86 potent HIV transcription inducers that release NFkB, AP1 and PTEFb (51, 52).
87 However, their applications have been limited by their toxicity and difficulty of
88 synthesis. Nonetheless, their promising profile, combined with the generation of
89 potent analogs, might support their clinical development as latency reversing agents
90 (LRAs) *in vivo* (53).

91 Based on the importance of epigenetics for HIV transcription, inhibitors of histone
92 deacetylase (HDACi) are believed to be promising LRAs (54-57). One of these,
93 Vorinostat, was safe and efficacious in promoting transcription of cell-associated HIV
94 RNA in CD4+ T cells, but no decrease in the number of infected cells was achieved.
95 Another pan-HDACi, Panobinostat, induced HIV transcription more efficiently than
96 Vorinostat. Intriguingly, the transient decline of total HIV DNA correlated with
97 stimulation of the innate immune system, mainly activation of NK and plasmacytoid
98 dendritic cells (pDC) (56). Thus, support from the immune system seems to be
99 needed to clear the latently infected cells, as reversion from HIV latency alone is
100 insufficient to induce cell death (58), most likely because of low viral production (59).
101 Moreover, impaired HIV-specific CTL responses (60, 61), CTL escape HIV variants
102 (62) in concert with the immaturity of DCs (63-65), emphasize the need of re-
103 enforcing the immune system, in particular, the HIV-specific CTLs, to deplete the
104 infected cells

105 Various promising strategies target the innate immune system that will eliminate
106 cells switching from latent to productive HIV infection. Among the most promising are
107 toll-like receptors (TLRs), such as TLR9 (66), TLR8 (67), and TLR1/2 (68). TLR7 on
108 DCs (69), in particular, has emerged as an approach to induce HIV transcription and
109 direct a cytotoxic immune response. Indeed, TLR triggering modulated DC activity, T

helper and macrophage polarization (70-72) and displayed various effects on HIV replication (73, 74). Notably, TLR7, 8 and 9 are expressed on DCs, and their stimulation resulted in DC-dependent changes of the microenvironment. TLR signaling could also act on the apoptosis sensitivity of immune and cancer cells (75). Altogether, TLR triggering is a promising multifactorial adjuvant to eliminate the latent reservoir. It induces HIV expression and antiviral cytokine production, which interferes with spreading infection as well as T- and NK-cell maturation, which might deplete HIV-infected cells.

Here we proposed that concomitant use of transcriptional enhancers and immune response inducers is a potent strategy for reactivating HIV replication. Acting on different transcriptional repression mechanisms is most likely key factor for efficient reversion of HIV latency (76, 77). We tested the hypothesis that Prostratin (acting directly on latently infected T-cells), in concert with TLR8ag (acting via DC), disrupts HIV latency (67) and might trigger the priming and restoration of antigen-specific immunity, through co-stimulatory molecules and IL-12p70 expression (72, 78, 79). Adding TLR8ag might lead to a Th1 supportive milieu crucial to clear the persistent quiescent reservoir *in vivo*. To explore this possible interplay, J-lat cells were co-cultured with monocytes-derived dendritic cells (MDDCs), representing the inflammatory DC compartment (80-82), and subsequently challenged with diverse compounds. The reactivation potency was evaluated using LTR-driven eGFP expression and the overall outcome by characterizing both players. In a second step, we verified this approach on primary cells from aviremic patients.

MATERIALS AND METHODS

Antibodies and reagents.

Prostratin (P0077), 5-aza-2'deoxycytidine (Aza-CdR, A3656), Azidothymidine (AZT, A2169) and Efavirenz (SML0536) were purchased from Sigma-Aldrich. Prostratin and Aza-CdR were used at 0.5 µM, AZT was used at 5 µM and Efavirenz at 50 nM. Rh-GM-CSF, rh-IL-4 and TNF (10 ng/ml) were obtained from Immunotools (#11343127, #11340017 and #113440047, respectively). TLR8ag (3M-002), used at 1 µM, was purchased from 3M-Pharmaceuticals (St. Paul, MN; 55144-1000). Finally, SAHA, obtained from Cayman (#10009929), was used at 1 µM unless otherwise stated in the figure legends. Blocking antibodies CD80 and CD86 (Biolegend, #305201, 305401) were applied at 5 µg/ml and Infliximab was from R&D Systems (AF-210-NA) and used at 1 µg/ml for the TNF blocking experiments. The ImmunoCult™ Human CD3/CD28/CD2 T cell activator (#10970) from Stemcell Technologies was used based on the manufacturer's instructions. Flow cytometry antibodies were purchased either from Pharmingen (i.e., active Caspase3-PE (#550821), CD80-PE (#557227), CD83-PE (#556855), CD86-PE (#555658), or Biolegend, as CTLA-4-APC (#349907), ICOS-PE/Cy7 (#313519), DC-SIGN-FITC (#330103), HLA-DR-APC (#307609), or from Beckman Coulter p24-RD1 (#6604667). Finally, acetylated lysine-9 of histone H3 (H3K9-ac) antibody was obtained from Abcam (Cambridge, UK, #10812). The signaling inhibitors library, kindly provided by Prof. B. Schaefer and mainly originating from AxonMedchem, Selleck and Sigma-Aldrich, was applied for 1 h, at a final concentration of 500 nM, on J-lat cells alone before adding the MDDCs in combination with the stimuli.

Cell culture.

J-lat, J1.1, ACH2 and 8E5 cells, obtained from NIH (#9848, #1340, #349 and #95, respectively), were cultured in R-10 medium (i.e., RPMI 1640 medium (BioWhittaker) supplemented with 10% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco). J-lat and J1.1 cells were both derived from Jurkat cells (83, 84). MDDCs were generated by 7 days of stimulation of monocytes, isolated from healthy donors with CD14 microbeads (Miltenyi, #130-050-201), with 1'000 biologically active units per ml of rh-IL-4 and rh-GM-CSF. J-lat or J1.1 cells (10^5) were seeded in a 96-well plate with or without 10^4 MDDCs and cultured/treated for 24 h. J-lat cells and MDDCs were separated in a 24-well plate by applying a transwell (Millipore, Millicell, PIHP01250) with a pore size of 0.4 µm. Blocking antibodies CD80 and CD86 were pre-incubated 1 h before adding J-lat and the treatments. Azide (0.09 %), included in the CD80 and 86 blocking antibodies was added to the mock control.

Patients

We had access to specimens from HIV-infected patients successfully treated by cART (<20 copies/ml, as measured by the Cobas® Amplicor technology, Roche) for more than 4 years (median 12.5 years) with a median of 584 CD4+T cells/µl. The use of these specimens was approved by the Ethics Committee of the University Hospital Zurich and informed consent was obtained from all HIV-1-infected individuals recruited. All experiments were performed in accordance with the relevant guidelines and regulations.

PBMC were isolated from 30 ml of blood by Ficoll (Axis-Shield PoC AS, Norway) gradient centrifugation, followed by CD8+ T-cell -depletion using microbeads (Miltenyi, #130-045-201), and CD14+ cell isolation as previously described. The

remaining PBMC were cryopreserved until MDDCs' differentiation, and CD4+ T cells were then indirectly isolated (Miltenyi, # 130-096-533). Limited blood volume and cell numbers restrained the experiment setup to 10^5 CD4+ T cells and 10^4 MDDCs per well in quintuplicate. These autologous co-cultures were stimulated with either Prostratin, TLR8ag, both combined, or anti-CD3/CD28/CD2 antibodies. AZT and Efavirenz were added to all the conditions. At day 2, 5, 7 and 9, 50 µl of supernatant per well was collected, pooled per donor and treatment, and was analyzed for HIV RNA copy numbers. Medium was replaced twice a week until the end of the experiment.

Flow cytometry.

J-lat clone 9.2, MDDCs or both combined were incubated with the cell-surface marker antibody at an optimized dilution in FACS buffer (PBS containing 2 mM EDTA, 0.1 % sodium azide and 10 % FCS) for 20 minutes at 4 °C. Cells were subsequently washed with FACS buffer and fixed with 1 % paraformaldehyde (PFA) in PBS until acquisition. Permeabilization kit (BD, cytofix/cytoperm, #554714) was used for intracellular staining for active Caspase-3, H3K9-ac and p24 antigen according to the manufacturer's instructions. Stained cells were acquired on a CyAn TM ADP Analyzer (Beckman Coulter), and data were analyzed using FlowJo (version V.10.0.8). We defined the live cells by the side scatter/forward scatter gate and then quantified the number of cells by the specific marker of interest.

Cytokine measurements.

Supernatants (SN) of stimulated MDDCs were collected at 6, 12, 24 and 36 h and subjected to quantification of TNF, MIP1α and IL12-p70. Human cytokines were

analyzed using a multiplexed particle-based flow cytometric cytokine assay (85). Cytokine kits were purchased from R&D Systems. The procedures closely followed the manufacturer's instructions. The analysis used a conventional flow cytometer (Guava EasyCyte Plus, Millipore, Zug, Switzerland). The values (pg/ml) obtained were normalized on the mock-treated control.

Viral RNA measurements.

HIV RNA was isolated from 250 µl culture supernatant using the QIAmp Viral RNA Mini Kit (Qiagen, #52906). Serial dilution of PBMC-propagated Yu2, quantified by Cobas® Amplicor technology (Roche), were simultaneously isolated and used as standards. Subsequently, viral RNA was reverse transcribed using the iScript™ Select cDNA Synthesis Kit (Bio-Rad, #170-8897) in combination with a gene-specific primer (0.25 µM reverse, TACTAGTAGTTCCTGCTATGTCACTTCC). HIV DNA was amplified using the Maxima Hot Start PCR Master Mix (Thermo Scientific, K1052), with 1 µM forward primer (5'-CAAGCAGCCATGCAAATGTTAAAAGA-3'), 0.3 µM probe (5'-FAM-TGCAGCTTCCTCATTGATGGT-BHQ1-3') and 1 µM of the above mentioned reverse primer. Cycling conditions were 95 °C for 4 min, and then 50 cycles of 95 °C for 5 sec, 55 °C for 5 sec and 60 °C for 30 sec. Reactions were performed on a Bio-Rad iCycler (170-8740) and analyzed with the IQ5 software (Biorad). Copy numbers (copies/ml) were adjusted to the initial volume of supernatant.

222 **Statistics**

223 The software GraphPad Prism Version 5.04 was used for doing statistics. The
224 statistical test employed is indicated in the legends. Two-tailed paired t-test was
225 performed on the individual replicates between parameters, otherwise stated. Mann-
226 Whitney test and ANOVA were used for the viral RNA peak value statistics. $P \leq 0.5$
227 was considered statistically significant.

RESULTS

Differential potency of LRA on J-lat clones alone or co-cultured with MDDCs

We first screened various J-lat clones (83) for their signal-to-noise ratios by looking at their LTR-driven eGFP expression when stimulated with Prostratin, Aza-CdR, or TNF ± Aza-CdR (Fig. 1A)). Several clones (i.e., clone 6.3, 8.4, 9.2 and 15.4) had good signal-to-noise ratios, and we eventually used clone 9.2 for all experiments.

We believe that the immune system and in particular myeloid dendritic cells (mDCs) are key players in HIV cure. They generate a microenvironment potentiating the effects of LRAs and enabling an HIV-specific CTL response. Thus, we designed a co-culture of latently infected T cells, represented by the J-lat clone 9.2 and MDDCs at a ratio 10:1. Without any exogenous stimuli, this set-up did not alter the reactivation latency background of J-lat 9.2 but tended to increase ICOS and CTLA-4 expression, pointing to a potential activation of J-Lat cells by the MDDCs (86, 87) (data not shown).

Then, we challenged several known LRAs, including PKCag, HDACi, and DNA methyltransferase inhibitor, and various TLR agonists (TLRag) for their ability to reverse latency in J-lat cells alone (Fig. 1B, upper panel) or co-cultured with MDDCs (Fig. 1B, lower panel). In J-lat monoculture, HIV was effectively induced by SAHA and TNF, as reported (88). Prostratin, Trichostatin A (TSA), Aza-CdR as well as TLR2, 4, and 8ag had modest or no effect. The lack of reversion in J-lat cells by TLRs is consistent with their low or absent levels of TLR2, 4 and 8 mRNA expression, which was not altered upon stimulation (data not shown). Strikingly, Prostratin in co-culture led to greater HIV reactivation than any other compound (Fig. 1B). These data underlined the importance of studying potential LRAs and their

combinations in a more complex system than pure T-cell cultures. Moreover, it prompted us to focus primarily on Prostratin as an adjunct to immune inducers that act via other immune cells, such as mDCs. Triggering TLR4 and 8 in co-culture also induced HIV transcription to a similar level as SAHA.

Prostratin achieved superior HIV latency reversion in co-culture, further enhanced by TLR8 agonist.

Prostratin showed a dose-dependent reversion of latency in J-lat 9.2 cells with moderate effects at 1 μM and minimal ones at 0.1 μM (Fig. 2A). Notably, the extent of reversion correlated with cell death rate (Fig. 2B), which is most likely mediated by viral cytopathicity and not due to drug-induced toxicity. The latter explanation is supported by the higher viability of the parental Jurkat cells when exposed to similar concentration of Prostratin, i.e., 71 % compared to 49 % in J-lat 9.2 (Fig. 2C).

Adding the immune inducer TLR8ag to Prostratin in the co-culture system resulted in much greater reversion of latency than the PKCag alone (Fig. 2D). This effect was also seen with other TLR agonists, such as TLR2 and TLR4 agonists (data not shown). Indeed, adding TLR8ag sensitized J-lat cells to lower concentrations of Prostratin without any additional toxicity (Fig. 2E). Furthermore, this combination resulted in higher eGFP mean fluorescence intensity (MFI), which suggests stronger HIV transcription and thus viral particle production (Fig. 2F). Additionally, we observed a plateau of efficacy at 0.5 μM of Prostratin, supporting this concentration for the subsequent use.

Effects of Prostratin and/or TLR8ag on other latently HIV-infected cell lines.

We wanted to corroborate the data obtained in other T-cell lines. We screened the latently infected T-cell lines, 8E5, ACH2 and J1.1. 8E5 cells produced continuously

HIV particles as measured by staining for intracellular p24 (data not shown). ACH2 cells showed close to 100% reversion of latency in response to Prostratin and TNF at the doses used for the J-lat cells 9.2 (data not shown). Thus, we did not pursue any additional experiments with these two cell lines.

The J1.1 cell monoculture reached a reversion of latency of 61.5 % ± 1.99 with TNF and 30.95 % ± 0.49 with Prostratin (Fig. 3). In the co-culture setup, the J1.1. cells were more responsive to Prostratin with 42.37 % ± 6.28 cells positive for intracellular p24 antigen (Ag) than in the monoculture. However, Prostratin in concert with TLR8ag resulted in a similar frequency of reverted cells as with TNF alone. Adding the TNF blocker Infliximab to the co-culture treated with Prostratin/TLR8ag recapitulated the findings found in J-lat cells; the remaining latency reversion was greater, although not significantly, than the one observed in co-cultures treated with Prostratin and Infliximab pointing to the causal role of other soluble factors or cell-cell contact herein. The effects observed with J1.1 were less prominent compared to J-lat 9.2 cells, at the doses established in the latter one.

TNF and cell-cell interaction are involved in the enhanced reactivation in co-culture.

Using transwells and Infliximab, we explored the mechanisms that led to the increased reversion of latency (Fig. 4A). Indeed, separation of the two cell types, as well as inhibiting TNF signaling drastically reduced the synergy of Prostratin and TLR8ag up to 70 %. A combination of transwells and Infliximab abrogated this positive interplay up to 95 %. The remaining latency reversing activity corresponded to the modest effect of Prostratin on J-lat cells. These findings underlined the major role of soluble factors and cell-cell contact for the reversion of latency in our model.

We verified that the dose of Infliximab added to the co-culture was sufficient to neutralize all TNF produced by MDDCs. We added increasing amount of TNF to the J-lat cells while keeping the dose of infliximab constant. 1 µg/ml of Infliximab inhibited the reversion of latency in J-Lat cells treated with 100 ng/ml of TNF (Fig. 4B), which is 5 fold more than the maximum of TNF released by TLR8ag stimulated MDDCs over 24 h (5-20 ng/ml, data not shown). This experiment substantiates the conclusion that TLR8ag stimulated MDDCs act also by other means, as cell-cell contact, than TNF on J-lat cells for reverting latency.

Short exposure of MDDCs to solely TLR8ag was sufficient to mediate potent eGFP expression in J-lat cells consistent with their rapid and efficient maturation (Fig. 4C). The signal was slightly lower than the control and implied a direct role of Prostratin in the activation/maturation of MDDCs. Conversely, MDDCs cultured for 1 h with Prostratin, followed by washing and adding J-lat cells with TLR8ag, resulted in a reduced response, pointing to the absolute need of Prostratin for J-lat reactivation (Fig. 4C). Finally, we explored whether supernatants from MDDCs would have any effect on latency reversion in J-lat cells. The cells were pre-treated for 2 h to achieve maximal stimulation, washed and further cultured without stimuli for 24 h. When the supernatant was transferred onto J-lat cells, we only observed background eGFP expression, explained by the unstable nature of the factors secreted by the MDDCs (Fig. 4C). Furthermore, Prostratin and TNF stimulation of J-lat cells or supernatant transfer of TLR8ag-treated MDDCs supplemented with Prostratin, led to a lower reversion of latency than Prostratin and TLR8ag added directly to co-cultures (Fig. 4D).

323 Altogether, these findings demonstrated the role of Prostratin, TNF and cell-cell
324 interaction in the enhanced latency reversion in co-culture.

325 Thus, multiple pathways must be stimulated for a sustained induction of NF-κB and
326 *de novo* synthesis of Tat, which is required for robust expression of elongated HIV
327 transcripts (89). Prostratin appears to be essential to initiate HIV transcription on J-lat
328 cells, which is further enhanced by the concomitant stimulation by TLR8-matured
329 MDDCs via TNF and co-stimulatory molecules.

330 **Prostratin and TLR8ag modulated the phenotypic and functional**
331 **characteristics of MDDCs but CD80-86 and ICOSL had no role in the enhanced**
332 **reversion of latency in co-cultures exposed to TLR8ag and Prostratin.**

333 We considered the phenotypic and functional changes of MDDCs essential for
334 understanding the superior latency reversion in response to TLR8ag and Prostratin,
335 and especially for further development of this approach. Prostratin led to an
336 adherent, constellation-like phenotype of MDDCs (data not shown) and induced
337 minor CD80 and CD83 expression at 24 h. In contrast, the percentage and MFI of
338 CD86 were substantially increased within 12 h and remained elevated over the entire
339 observation period of 36 h (Fig. 5A upper panel; data not shown). Further, Prostratin
340 promoted TNF production as reported (45) and a transient MIP1α secretion at 12 h
341 but without any induction of IL-12p70 (Fig. 5B). We also observed increased HLA-DR
342 expression concomitant with DC-SIGN downregulation (Fig. 5C). Therefore,
343 Prostratin might restrain spreading infection through DC-SIGN reduction and
344 enhancing antigen presentation by upregulating HLA-DR. These findings highlighted
345 the pleiotropic effects of Prostratin in MDDCs that might contribute to a less
346 permissive micro-environment for HIV dissemination.

347 As expected, TLR8ag triggered rapid upregulation of maturation markers (Fig. 5A,
348 lower panel) and sustained production of TNF (Fig. 5B). Importantly, with the
349 increasing secretion of IL-12p70, TLR8 stimulation might license mDCs to rescue the
350 adaptive immune response from HIV-associated exhaustion. In addition, Prostratin
351 and TLR8ag did not antagonize each other (Fig. 5C).

352 Thus, Prostratin and TLR8ag induced a functional maturation of MDDCs, optimal for
353 the reversion of latency. Furthermore, the recruitment of immune cells by MIP-1α, the
354 reduced capture and transmission of HIV virions by mDC, in combination with IL-
355 12p70 secretion and enhanced MHCII antigen-presentation, might promote a Th1
356 response
357 that contributes to an HIV-restrictive environment.

358 Next, we tried to identify the receptor-ligand pair involved in the cell-mediated
359 enhanced reversion of latency. We focused primarily on the CD80/86-CD28 axis
360 since these costimulatory molecules were substantially increased on MDDCs treated
361 with Prostratin and TLR8ag (Fig. 5A). Strikingly, suppressing the CD80/86 axis with
362 neutralizing antibodies (NAb) further increased reversion of latency in this setting
363 (Fig. 5D). We explain this result by a preferential interaction of CD80/86 with the
364 inhibitory receptor CTLA-4 (Fig. 5E). Therefore, its blocking potentially removed
365 some inhibitory pressure on the signaling pathways triggered by Prostratin and
366 TLR8ag.

367 The CD28 family member, ICOS, was also significantly up-regulated in Prostratin
368 treated co-culture (Fig. 5.E), irrespective of TLR8ag. However, blocking ICOSL with
369 up to 10 ug/ml of NAb did not reduce eGFP expression, excluding ICOSL as relevant
370 costimulatory molecule for latency reversion (data not shown).

Prostratin triggered moderate apoptosis in J-lat cells and had no deleterious effect on MDDCs.

LRAs may induce cell death in the target cells by reversion of latency but also in uninfected cells. Prostratin alone induced moderate apoptosis in J-lat cells, as quantified by active Caspase-3 (Fig. 6A). Prostratin or TLR8ag displayed no toxicity on MDDCs (Fig. 6B). Similarly, the combined treatment of Prostratin and TLR8ag on co-culture did not show any additive or synergistic detrimental effects on J-lat cells (Fig. 6C). Importantly, apoptosis induction was positively correlated with eGFP expression in J-lat cells (Fig. 6D), but was lost in the co-culture setup (Fig. 6E). Consistent with its anti-tumor activity, SAHA triggered a high level of apoptosis in J-lat cells, in MDDCs as well as in co-cultures (Fig 6A, B, C). SAHA's apoptosis rate was even higher than its effect on reversion of latency, especially in co-culture (Fig. 6D, E) highlighting its potential cellular toxicity.

Prostratin and TLR8ag stimulated co-cultures increased moderately but significantly the chromatin accessibility, enabling HIV transcription.

Since relaxation of the chromatin is crucial for transcription factors access and for initiation of efficient HIV transcription, we examined the global chromatin relaxation in response to our treatments, by measuring the histone H3 lysine 9 acetylation (H3K9-ac). Prostratin with and without TLR8ag showed no effect on chromatin relaxation. in J-lat cells alone (Fig. 7). In contrast, co-cultures stimulated with both compounds led to a significant chromatin relaxation compared to the mock treated co-culture. The effect on chromatin relaxation overall was not very prominent, but a given signaling pathway, e.g., TLR8, most likely alters sparse regions of the chromatin (90). Furthermore, the one shot analysis done at 24 hours will not mirror the dynamic

changes over time (91). Therefore, the minor changes observed on the overall chromatin remodeling may be well sufficient for enhanced HIV transcription in the context of combined treatment with Prostratin and TLR8ag. In contrast, SAHA drastically modified the acetylation status of H3K9 (up to 70 %), which was not correlate with the reversion of HIV latency.

SYK, MEK, PKCβ mediated the enhanced reversion of latency by Prostratin and TLR8ag in co-culture.

To assess the signaling pathways involved, we used a library of inhibitors (kindly provided by Prof. Schäfer). In Prostratin/TLR8ag treated co-culture, inhibition of SYK, MEK (1-2) and PKCβ resulted in the most striking loss of latency reversion (Fig. 8A). This finding is consistent with the central role of SYK in the immune-receptor pathway in hematopoietic cells. Furthermore, activation of the PKC pathway via Prostratin, was demonstrated by the reduced eGFP expression upon PKCβ inhibition. As SYK and PKC signal through MEK/ERK/NFκB, blocking MEK interferes with both pathways.

In co-culture treated with Prostratin, SYK, PKCβ and MEK were also involved (Fig. 8B, red bars), as well as PDK1±TBK1 and GSK3. The PI3kinase activation appeared not to have any effect on reversion of latency in this setup (Fig. 8B, blue bar). Notably, the serine/threonine kinase TBK1 and GSK3 induce TLR-dependent NFκB nuclear translocation and TNF synthesis (92), pointing to the prominent contribution of NFκB. Inhibition of PKCβ and PDK1 in co-culture, only modestly reduced reversion of latency upon Prostratin and TLR8ag treatment, as compared to Prostratin alone (Fig. 8B, C). We speculated that there were extensive cross-signaling events, creating some redundancy and thus lessening the role of PKCβ and PDK1 in the

overall response. We could neither detect any involvement of AKT, a downstream substrate of PDK1, nor JNK and p38, involved in TLR and TNF signaling (Fig. 8B, C). This phenomenon might be explained by a SYK-dependent inhibition of JNK and p38 phosphorylation (93). Interestingly, inhibiting phospholipase C (PLC) led to an enhanced HIV transcription, especially in Prostratin-treated J-lat monoculture (Fig. 8B, C).

Latency reversion in co-cultures with cells from aviremic HIV-infected individuals in response to either Prostratin ± TLR8ag or CD3/CD28/CD2 stimulation.

Eventually, we examined our strategy using primary cells from HIV-infected individuals with suppressed HIV RNA (Table 1). We performed a co-culture of autologous CD4+ T cells and MDDCs. By adding the RT-inhibitors we detected exclusively the virions emerging from the latent reservoir and thereby we were able to estimate the potency of the various compounds.

The limited number of specimens assessed and the rather large donor- and time dependent variability of the assay results prompted us to compare the average of mock-treated samples ($p=0.23$ between the donors, ANOVA) with all treated samples irrespective of the compound and time point. We observed a significant increase in viral RNA production upon simulation as compared to the mock condition ($p=0.0052$) (Fig. 9A). In most cases the increase was most prominent at day 5 after stimulation. Notably, we observed donor-dependent sensitivity to distinct LRAs, which is a well-known phenomenon (94) (Fig. 9B). Even the specimens treated with anti-CD3/CD28/CD2 (positive control) did not result in uniform latency reversion (*i.e.*, donor 4 and 8). We also observed distinct magnitudes in latency reversion with the

various LRAs, e.g., in donor 6 and 9, Prostratin+TLR8ag was more potent than either compounds alone. At contrary, in donor 4 and 7, Prostratin+TLR8ag had not effect. We explain this heterogeneous result by donor-specific responsiveness to LRAs, reservoir size and underlying latency mechanisms.

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DISCUSSION

Here, we investigated the efficacy of compounds directly targeting latently infected T cells in concert with innate immune system stimulation. We found superior reversion of latency in a co-culture of T cells and MDDCs, *in vitro* and *ex vivo,* using cells from aviremic HIV-infected individuals, when exposed to the PKC agonist, Prostratin, and a TLR8 agonist. Soluble factors, in particular TNF, and cell-cell contact contributed to the superior reversion of latency. Notably, the TLR8-mediated IL12p70 increased secretion might be crucial for restoring antigen-specific CTL activity (95, 96). Therefore, such a combined approach might be very promising in latency reversion and conceivably in restoring adaptive immune responses needed to eliminate latently infected T-cells.

The study of latently infected cells is challenging. Their frequency is quite low, contain a fraction of replication-competent but hardly inducible proviruses (*i.e.,* $1/10^5$–10^6 T cells) (1, 97), and they cannot be segregated from their uninfected counterpart. Moreover, patients' diversity, latently infected cells features as well as limited blood or tissues sampling impede screening of LRAs. Therefore, latency T cells models remain useful for challenging new "shock and kill" strategies. Various latency T-cell models have been generated (88), but they lack the influence of neighboring cells, such as DCs, in the nature and nurture of latently infected cells. We established a co-culture model consisting of the HIV latently infected T-cell line, J-lat, and MDDCs. MDDCs resemble inflammatory DCs (82) and are involved in early pathogen-specific T-cell responses (98). Thus, they may act indirectly on latently infected cells upon activation. We used this model to determine if triggering the DC compartment in

concert with LRA had additive or synergistic effects over targeting latently infected cells alone.

We first characterized the latency reversion features of various J-lat clones to established LRA (Fig. 1A). We found high LRA sensitivity disparity between the clones, that argues or clonal variegation leading to multiple refractory mechanisms to latency reversion (99). Moreover, maximum latency reversion was hardly achieved, These intrinsic features were also observed in primary cells (97).

Then, we compared the potency of several known LRA on J-lat cells clone 9.2 alone or co-cultured with MDDCs (Fig. 1B). SAHA stood out for its ability to reverse HIV latency in J-Lat cells. In the co-culture model, however, this effect was partially lost. This observation is consistent with the immunosuppressive activity of HDAC inhibitors (100) and emphasizes the need to investigate LRA in more complex settings than only latently infected cell lines. Prostratin, a non-tumorigenic phorbol ester, showed some modest latency reversing activity in J-lat cells alone, as reported (51, 76). Notably, Prostratin reactivates latent HIV and restricts HIV replication via i) transient activation of several PKC isoforms (51), leading to NFκB and Cyclin T1 availability (52), both being limiting factors for latency reversion, ii) cell-surface downregulation of CD4 and CXCR4 and DC-SIGN (43, 101) (Fig. 5C) and upregulation of the HIV restriction factor p21 (102, 103). Intriguingly, in the co-cultures, Prostratin exhibited a substantially increased number and intensity of eGFP-expressing cells, but the level of apoptosis was similar to J-lat cells alone (Fig. 1B). In contrast to SAHA, adding MDDCs reinforced substantially the LR effects of Prostratin, leading to MDDCs maturation and enhancing latency reversion.

493 We primarily tested the TLR agonists 2, 4 and 8 for their ability to stimulate the
494 MDDCs. They had no effect when added to J-lat cells alone (Fig. 1B), consistent with
495 the lack of TLR2, 4 and 8 mRNA expression even upon treatment (data not shown).
496 In the co-culture system, TLR 4 and 8ag displayed potent activity in inducing HIV
497 expression as judged by the eGFP expression in J-lat cells, but the TLR2ag had only
498 a modest effect (Fig. 1B). TLR4 also triggers a prominent cytokine storm that would
499 exclude its clinical application. In contrast, TLR7 and TLR7/8 agonists have been
500 successfully and safely applied to patients with hepatitis B (104) and C (105) infection
501 and thus are rationale candidates to test in concert with Prostratin. Notably, TLR8ag
502 beneficially affected various steps leading to the generation of an efficient adaptive
503 immune response (67, 74, 106). Importantly, Prostratin with the TLR8ag led to a
504 significantly larger breadth and intensity of HIV latency reversion than Prostratin
505 alone (Fig. 2D, F). These findings suggested a reinforcement of signaling events,
506 which might target and reactivate various cell types, harboring diverse HIV
507 transcription blocks *in vivo.*
508 We choose another latently HIV infected T-cell line, namely, the J1.1 cell line (84)
509 which was obtained by limiting dilution of HIV-1 LAV infected Jurkat E6 Cells, to
510 corroborate the data observed with the J-lat cells. The J1.1 cell line, was very
511 responsive to TNF and to the combination of Prostratin and TLR8ag, at the doses
512 established in the J-lat clone 9.2 (Fig. 3). We observed similar effects in co-culture
513 but they were less prominent compared to the J-lat clone 9.2. The differing reactivity
514 to LRAs between these cell lines are consistent with the intrinsic sensitivity of most
515 cell models to particular stimuli, as stated by Spina et al. (88).

516 TNF plays certainly a key role in the reversion of HIV latency in this setup.
517 However, reversion of latency was still observed when excess of NAb against TNF
518 was added (Fig. 4A, B). We found that cell-cell contact contributes to the reversion of
519 latency in co-culture. Indeed, the transfer of supernatant from TLR8ag-stimulated
520 MDDCs onto Prostratin-treated J-lat cells and the stimulation of J-lat cells with
521 Prostratin and TNF, did not reach the level of reversion observed in the co-culture
522 setup (Fig. 4D). Thus, soluble factors and cell-cell contact contributed to the overall
523 effects observed.

524 Further detailed characterization revealed that both compounds promoted
525 phenotypical and functional maturation of MDDCs, the effects being more prominent
526 in response to the TLR8ag than to Prostratin (Fig. 5). Secreted IL12p70 and CD83
527 engagement might drive a Th1 immune response through i) NK activation, ii) naive
528 CD8+ T-cell priming, iii) expansion and survival of antigen-specific CD8+ T memory
529 cells (78, 107-110), and iv) restoration of exhausted CD8+ T cells (95, 96).
530 Intriguingly, the maturation markers, CD80 and CD86 were most likely interacting
531 with the inhibitory molecule CTLA-4, as their neutralization resulted in enhanced
532 viability and eGFP expression. Indeed, CTLA-4 was induced on J-lat cells when
533 adding MDDCs (Fig. 5E). Hence, blocking CTLA-4, together with Prostratin and
534 TLR8ag treatment, might potentiate the shock and kill strategy (111). Similarly, ICOS
535 was upregulated in co-culture upon Prostratin-mediated T-cell activation. However,
536 increasing concentrations of ICOSL blocking antibodies did not reverse latency (data
537 not shown), despite ICOSL expression by MDDCs (112). Of note, ICAM1,
538 upregulated by TNF signaling on DCs, was recently associated with HIV reactivation
539 in proliferating CD4+ T cells, in a similar system (113). Since Jurkat cells do not

multimerize LFA-1, crucial for its activation and interaction with ICAM1, we did not challenge this axis (114).

Activation and latency reversion might proceed in parallel with cell death of the target, as well as in bystander cells. Therefore, promising LRAs should be evaluated for their potential side effects (Fig. 6). As expected, SAHA increased active Caspase-3 up to sevenfold in J-lat cells, as reported (115, 116). Surprisingly, we observed a similar induction in MDDCs. In contrast, Prostratin induced a threefold increase of active Caspase-3 in J-lat cells and none in MDDCs. Importantly, adding the TLR8ag did not enhance the Prostratin-mediated apoptosis.

In sum, the combination of stimuli chosen exhibited no detrimental effect on both cellular players, and the enhanced HIV transcription observed without increasing apoptosis might be optimal for providing an antigenic boost to CTLs.

We next wondered if the combinatorial stimulation of J-lat cells and MDDCs would remove epigenetic blocks by looking at the acetylation status of lysine 9 on histone H3 (H3K9-ac). Upon cell stimulation, transcription factors recruit histone acetyl transferase (HAT) and nucleosome-remodeling complexes. They allow the binding of the transcriptional machinery through chromatin relaxation (117-120). Prostratin alone did not induce any H3K9 acetylation of the overall chromatin 24 h after stimulation while given together with TLR8ag, it resulted in a significant relaxation of the chromatin (Fig. 7). This result endorses the highly dynamic and tailored signaling-induced chromatin remodeling paradigm. Thus, the combination of those compounds acted at multiple levels to reverse latency, through chromatin accessibility, NFκB and PTEFb induction in this model (121). On the other hand, SAHA treatment led to a global acetylation pattern, which was not correlated with reversion of latency, highlighting the heterogeneity of the latency mechanisms responsible for HIV latency.

565 Using a library of signaling inhibitors, we demonstrated here that blocking the
566 non-receptor kinases SYK and MEK drastically reversed the effects on latently HIV-
567 infected cells, triggered by Prostratin and TLR8ag (Fig. 8A). Moreover, their signaling
568 appeared crucial for all treatment in J-lat alone as in co-culture, indicating their
569 activation by Prostratin (Fig. 8A, B). SYK is a key downstream molecule in various
570 immuno-receptors signaling events, in pre-T cells, mature B cells (93, 122, 123) and
571 in malignancies (124, 125), e.g., J-lat cells. MEK, belonging to the MAPK pathway,
572 bridged the signaling events induced by Prostratin to latency reversion in J-lat cells
573 and in co-culture.
574 Various PKC isoforms translocate within the plasma membrane upon Prostratin
575 stimulation, such as PKCβ (51). Indeed, we observed a significant, but not complete,
576 contribution of PKCβ on latency reversion in J-lat cells. This highlighted the role of
577 others PKC isoforms, such as novel PKC (e.g., δ,ε,η,θ), that participated in the
578 overall response, through NFκB and PTEFb release (126). Of note, Prostratin-treated
579 co-cultures showed stronger PKCβ implication than J-lat alone, arguing for multiple
580 effects of Prostratin on both cell types (Fig. 8B). Addition of TLR8ag in the co-culture
581 drastically reduced the implication of PKCβ, TBK1 and PDK1 in the ultimate
582 outcome, pointing to the collaborative and redundant signaling events involved in this
583 setup (Fig. 8C). Moreover, the downstream effector within the TLR pathway, TBK1
584 (127), which participate in TNF production, demonstrated its relevance only in the co-
585 culture setup. We speculate that the TLR8-mediated MDDCs maturation and the
586 subsequent interaction with J-lat cells reduced the involvement of PKCβ, TBK1 and
587 PDK1 in the overall response, highlighting collaborative signaling events.
588 Surprisingly, inhibition of phospholipase C reinforced HIV reactivation in all the setup

(Fig. 8A, B). This may reflect its interaction with SYK through LAT in T cells (128, 129), a change in signaling homeostasis or an unspecific effect of the inhibitor.

Finally, we confirmed the relevance of the approach investigated in primary cells from aviremic patients. First, we demonstrated that latency reversion in response to treatments was not just a stochastic event since the compounds were clearly superior to the mock control (Fig. 9A). Second, the LRAs' potency was donor-dependent. Not even the positive control (anti-CD3/CD28/CD2) reversed latency uniformly (Fig. 9B). We also observed striking differences in the magnitude of latency reversion between compounds and donors. Actually, the extent of viral RNA production upon stimulation might be a crucial parameter for achieving the depletion of these cells (59). In particular, Prostratin+TLR8ag excelled in higher latency reversion in two donors than either Prostratin or TLR8ag alone. However, Prostratin+TLR8ag had no effect in two others; one of those was not reactivated even by the positive control.

Several parameters might affect the sensitivity of primary latently infected cells to a given LRA, such as the number of latently infected cells, the CD4+ T-cell subset, the epigenetic status as well as the presence of inhibitory receptors (130). Since we had been working with plain PBMC and not enriched lymphocytes and monocytes, obtained with leukapheresis, we might indeed encounter the risk that in some specimens the number of latently infected cells were too low or even absent. The number of latently infected cells is very small in human with estimated 1 to 60 provirus containing cells per million of CD4+ T cells (1, 97). Thus, by using 0.5 million of CD4+ T cells per condition, we estimated to have between 0.5 to 30 latently infected cells per condition. This could explain part of the variability observed, although HIV reactivation was detected in all the donors.

Another variable which may affect the outcome is the response of the *ex vivo* generated MDDCs to the TLR8ag and their interplay with autologous CD4+ T cells, as well as their survival in the co-cultures. Nevertheless, the data generated are very promising and should encourage future studies exploring combination therapies of immunomodulators and LRAs.

In conclusion, we established a novel *in vitro* co-culture system for testing compounds that mediate latency reversion. We showed that the synergistic efficacy of Prostratin and TLR8ag in co-culture can remove various repressive latency mechanisms through concomitant signaling events and confirmed its applicability to primary cells.

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Contributions

MAR and RFS elaborated the concept of this study, MAR did all of the experimental work, ES executed some experiments and gave conceptual inputs, and MAR and RFS wrote the manuscript.

Financial disclosure

n.a.

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Legends:

Fig. 1. Latency reversing agents demonstrated differential potency on J-lat cell clones or in co-culture of J-lat clone 9.2 with MDDC at a 10:1 ratio.

A) 10^5 J-lat clones were stimulated for 24 h with Prostratin, TNF with or without Aza-CdR in R-10 medium. The viability of treated J-lat clones (upper panel) was determined by the FSC/SSC characteristics. Reversion of latency (lower panel) was assessed by eGFP quantification, using flow cytometry, *n*=3, SD. B) J-lat cells clone 9.2 (upper panel) were treated for 24 h and analyzed for their viability and eGFP expression. TNF treatment represented the positive control, *n*=4, SEM. Co-culture of J-lat clone 9.2 with MDDCs, at a ratio 10:1 (lower panel), were similarly analyzed. CD40L designated the positive control for the co-culture setup, *n*=6, SEM (two-tailed paired t-test: **, 0.0072). The left Y axis depicted the viability and the right Y axis, the latency reversion. TNF: 10 ng/ml; CD40L: 50 ng/ml; Prostratin: 0.5 µM; TSA: 0.1 µM; SAHA: 10 µM; Aza-CdR: 0.5 µM; TLR2ag: 100 ng/ml; TLR4ag; 20 ng/ml; TLR8ag: 1 µM.

Fig. 2. Prostratin achieved superior HIV latency reversion in co-culture system, and was enhanced by TLR8 agonist.

A) J-lat cells 9.2 were stimulated for 24 h with different concentrations of Prostratin. The percentage of reactivated cells was quantified by LTR-driven eGFP expression using flow cytometry and depicted on the right Y axis. Viability was illustrated on the left Y axis, n=3. B) Correlation of the eGFP expression with J-lat cells viability (two-tailed Pierson correlation, n=3; SEM). C) Dose-dependent toxicity of Prostratin on parental Jurkat T cell (two–tailed paired t-test: *, p=0.033 and 0.0119, for 0.1 µM and 0.5 µM, respectively, when compared to mock; **, p=0.0053; n=4; SEM). D) Co-culture of J-lat cells 9.2 and MDDCs at a ratio 10:1, stimulated with increasing Prostratin concentrations with or without TLR8ag at 1 µM. Addition of TLR8ag significantly increased the reactivation potency of Prostratin (two-tailed paired t-test; **, p=0.0054; ***, p=0.0004; ****, p=<0.0001; n=6; SEM). E) Representative viability of J-lat cells from Figure 2D. F) Representative mean fluorescence intensity (MFI) of the eGFP expression by J-lat cells in the setup 2D; **, p=0.0098; ***, p=0.0007; ****, p=<0.0001; n=6; SEM. EGFP, a surrogate marker for viral protein production, was monitored using flow cytometry and viability based on the FSC/SSC characteristics.

Fig. 3. Effects of Prostratin and/or TLR8ag on J1.1 cell lines alone or co-cultured with MDDCs at a 10:1 ratio.

1105 10^5 J1.1 cells (blue bars, *n*=3) alone or in co-culture with MDDCs (10^4) (red bars,
1106 *n*=9) were stimulated for 24 h with Prostratin at 0.5 µM, TNF at 10 ng/ml and TLR8ag
1107 at 1 µM with or without 1 µg/ml of Infliximab in R-10 medium. Reversion of latency
1108 was assessed by intracellular p24 staining and quantified by flow cytometry, two-
1109 tailed paired t-test; *, p=0.0359; ****, p=<0.0001, SEM.

1110

1111 **Fig. 4. TNF and cell-cell interaction are involved in the enhanced reactivation**
1112 **observed in co-culture.**

1113 A) Reactivation potency of Prostratin and TLR8ag in co-culture, using a 0.4 µm pore
1114 transwell, 1 µg/ml Infliximab (TNF inhibitor), and their combination after 24 h of
1115 culture, *n*=6, SEM. The percentage of eGFP expressed was normalized on the
1116 reactivation of co-culture treated with 0.5 µM Prostratin and 1 µM TLR8ag per
1117 experiment (two-tailed paired t-test on the normalized value; **: 0.0093 for the
1118 transwell; **: 0.002 for the Infliximab; ***: 0.0003). B) Potency of 1 µg/ml of Infliximab
1119 to inhibit TNF-mediated latency reversion, assessed on J-lat cells clone 9.2 with
1120 increasing concentration of TNF (ng/ml), *n*=5, SEM. C) Upper panel: effect of 1 h
1121 TLR8ag pre-treatment of MDDCs, followed by washing and co-culture with J-lat cells
1122 9.2, in presence of Prostratin. Middle panel: Effect of 1 h Prostratin pre-stimulation of
1123 MDDCs, followed by washing and co-culture with J-lat cells 9.2, in presence of
1124 TLR8ag. Lower panel: 2 h TLR8ag pre-treatment of MDDCs, washed and cultured for
1125 24 h in R-10 medium. Transfer of the supernatant onto J-lat cells and further cultured
1126 for 24 h. Percentage of eGFP expressed was normalized on the reactivation of co-
1127 culture treated with 0.5 µM Prostratin and 1 µM TLR8ag, *n*=6, SEM. SN: supernatant,
1128 (two-tailed paired t-test on the normalized value; *: 0.0342; **: 0.0021; ****: <0.0001).

Normalized per experiment. D) Comparison of the latency reversion upon treatment, between j-lat alone (*n*=10), supplemented with the SN from 1 h or 2 h TLR8ag-stimulated MDDCs (*n*=14) and in co-culture with MDDCs (*n*=14). The green dashed line represented the latency reversion induced by the combination of Prostratin and TNF on J-lat cells. TNF was used at 10 ng/ml. The upper purple dashed line highlighted the enhanced latency reversion when co-culture was treated with Prostratin and TLR8ag. Addition of Infliximab did not abolish this latency reversion as depicted by the lower purple dashed line.

Fig. 5. Prostratin and TLR8ag modulated the phenotypic and functional characteristics of MDDC but CD80-86 and ICOSL had no role in the enhanced reversion of latency in co-cultures exposed to TLR8ag and Prostratin.

A) Upper panels: Kinetics of 10^5 MDDCs stimulated with Prostratin, *n*=11, median. Lower panels: Kinetics of MDDC treated with TLR8ag, *n*=8, median. The percentage of CD80-, 83-, and 86-expressing cells was monitored up to 36 h by flow cytometry and fluorescent antibodies. B) Fold-change of TNF, MIP1α and IL-12p70 concentrations in the supernatants of treated MDDCs with TLR8ag or Prostratin, *n*=6, SEM. C) Fold-change of mean fluorescence intensity (MFI) of DC-SIGN and HLA-DR to mock-treated MDDCs (two-tailed paired t-test; **, p=0.0021 and 0.0045, respectively, *n*=9, SEM). D) Neutralizing antibodies (NAb) against CD80, 86 at 5 µg/ml each and/or Infliximab at 15 µg/ml were applied to co-cultures stimulated with Prostratin and TLR8ag for 24 h. Viability, depicted on the left Y axis, and the reactivation, right Y axis, from J-lat cells 9.2 were normalized on the co-culture treated with Prostratin and TLR8ag (two-tailed paired t-test; *, p=0.0191; ***, p=0.0001; n=9, SEM). Note that 95% of J-lat cells expressed CD28 (data not shown).

1154 E) Expression comparison of CTLA-4 (*n*=6) and ICOS (*n*=8) in J-lat cells alone (left
1155 panel) or in co-cultures treated (right panel), *n*=6, (two-tailed paired t-test; *: 0.0131;
1156 ****, p<0.0001; *n*=8, SEM). Prostratin and TLR8ag concentrations were identical as in
1157 Figure 4.
1158
1159 **Fig. 6. Prostratin triggered moderate apoptosis in J-lat cells and shows no**
1160 **deleterious effect on MDDC.**

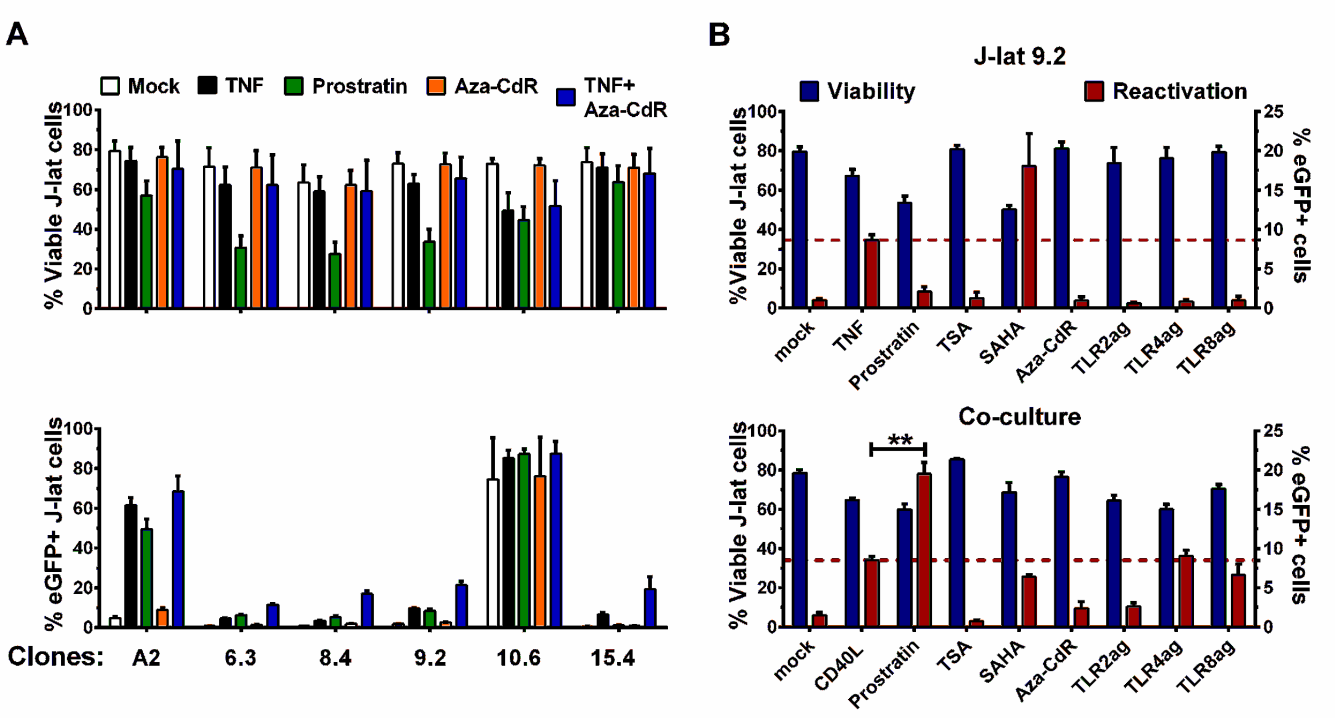
1161 The percentage of cells harboring an active Caspase-3 upon treatment is depicted.
1162 A) J-lat cells, *n*=5. B) MDDCs, *n*=3. C) J-lat cells in co-culture, *n*=6. D) Correlation of
1163 activated Caspase-3-positive J-lat cells, left Y axis, and corresponding eGFP
1164 expression, right Y axis, upon treatment (two-tailed Pierson correlation; *n*=4). E)
1165 Percentage of active Caspase-3 in J-lat cells, left Y axis, and corresponding eGFP
1166 expression, right Y axis, in co-culture, *n*=9. Prostratin and TLR8ag concentrations
1167 were identical as in Figure 3. SAHA was used at 1 μM and TNF at 10 ng/ml, SEM.
1168
1169 **Fig. 7. Prostratin and TLR8ag stimulated co-cultures increased moderately but**
1170 **significantly the chromatin accessibility, enabling HIV transcription**

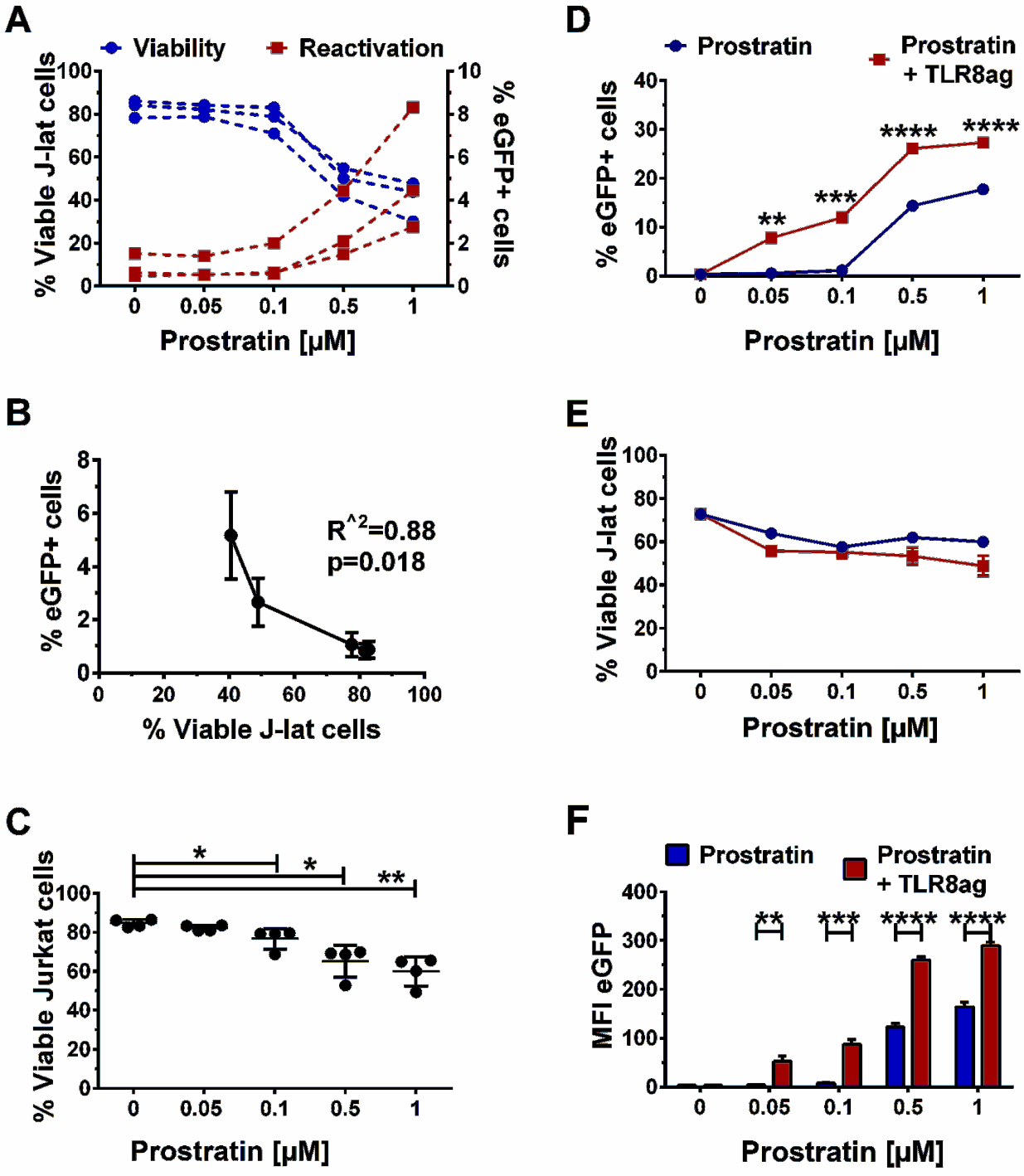
1171 Percentage of viable J-lat cells (blue line), eGFP expression (green line) and
1172 acetylated lysine 9 of histone H3 (H3K9-ac, red line) of treated J-lat cells alone (left
1173 panel, *n*=3) or in co-culture (right panel, *n*=9), SEM, (two-tailed paired t-test; **:
1174 0.0025). The concentration of stimuli was identical to the Figure 6.
1175
1176 **Fig. 8. SYK, MEK, PKCβ mostly mediated the enhanced reversion of latency by**
1177 **Prostratin and TLR8ag in co-cultures.**

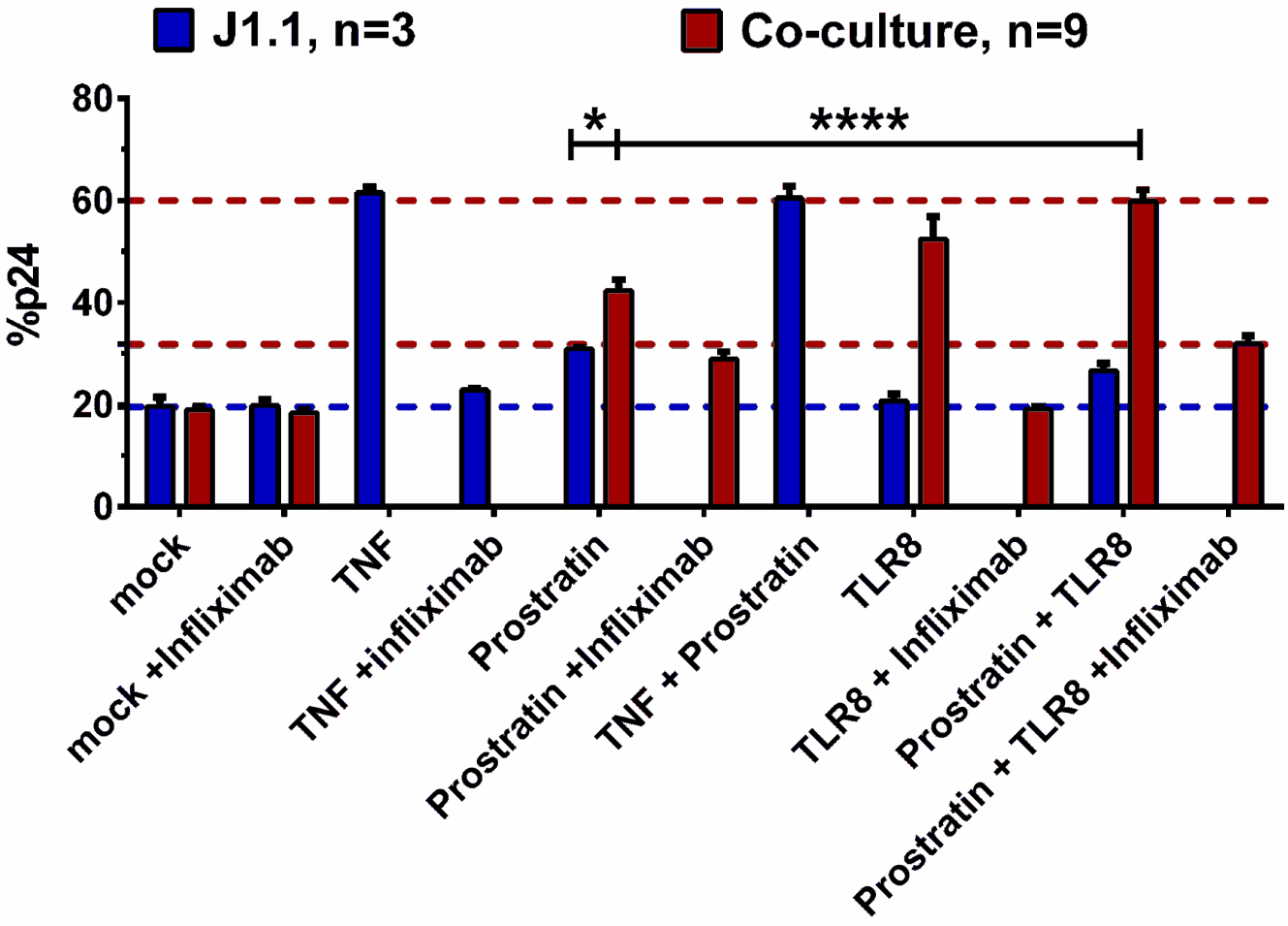
Reduction of eGFP observed when J-lat alone (blue) or in co-culture (red) were pre-treated for 1 h with 500 nM of signaling pathways inhibitors. The dashed lines represented the maximal reactivation obtained upon stimulation without the inhibitors. A) Percentage of viability, left Y axis, and eGFP expression, right Y axis, of J-lat in co-culture, pre-treated with kinase inhibitors, subsequently stimulated with Prostratin and TLR8ag for 24 h (two-tailed paired t-test; ****, p<0.0001; ***, p=0.0002 for MEK and 0.0003 for PKCβ; **, p=0.0044; *, p=0.0294; *n*=6, SEM). B) Detailed inhibition of latency reversion, upon Prostratin treatment, for every inhibitor used. C) Detailed inhibition of latency reversion, upon Prostratin and TLR8ag stimulation, for every inhibitor used (SYK, MEK, PKCβ, TBK1 and GSK3 from co-culture (red bars) already presented in Fig. 8A). The effects of the inhibitors on J-lat 9.2 monoculture are depicted with the black bars and on the co-culture with the red bars, *n*=3 for J-lat and *n*=6 for the co-culture, SEM.

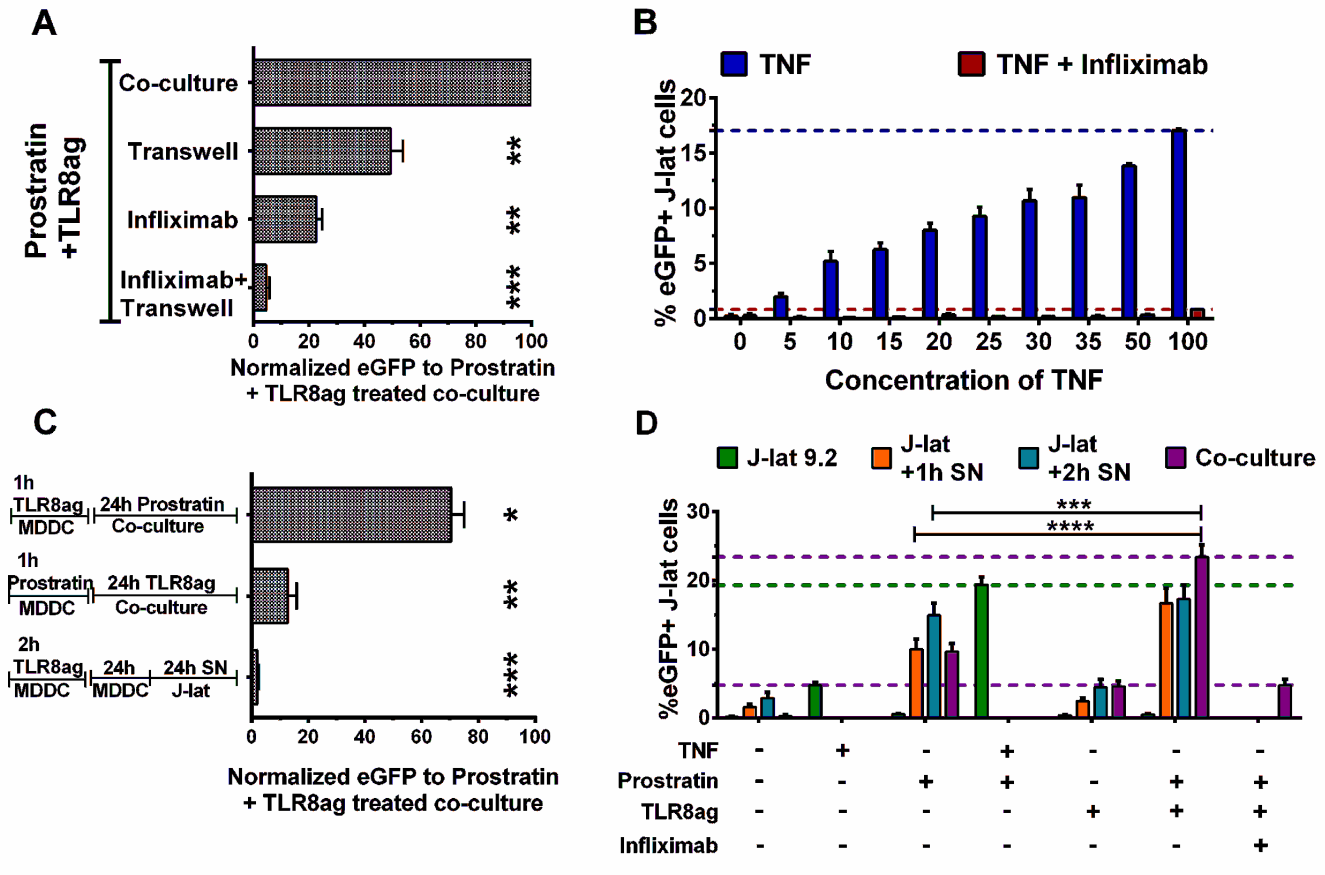
Fig. 9. Latency reversion in co-cultures with cells from aviremic HIV-infected individuals in response to either Prostratin ± TLR8ag or CD3/CD28/CD2 stimulation

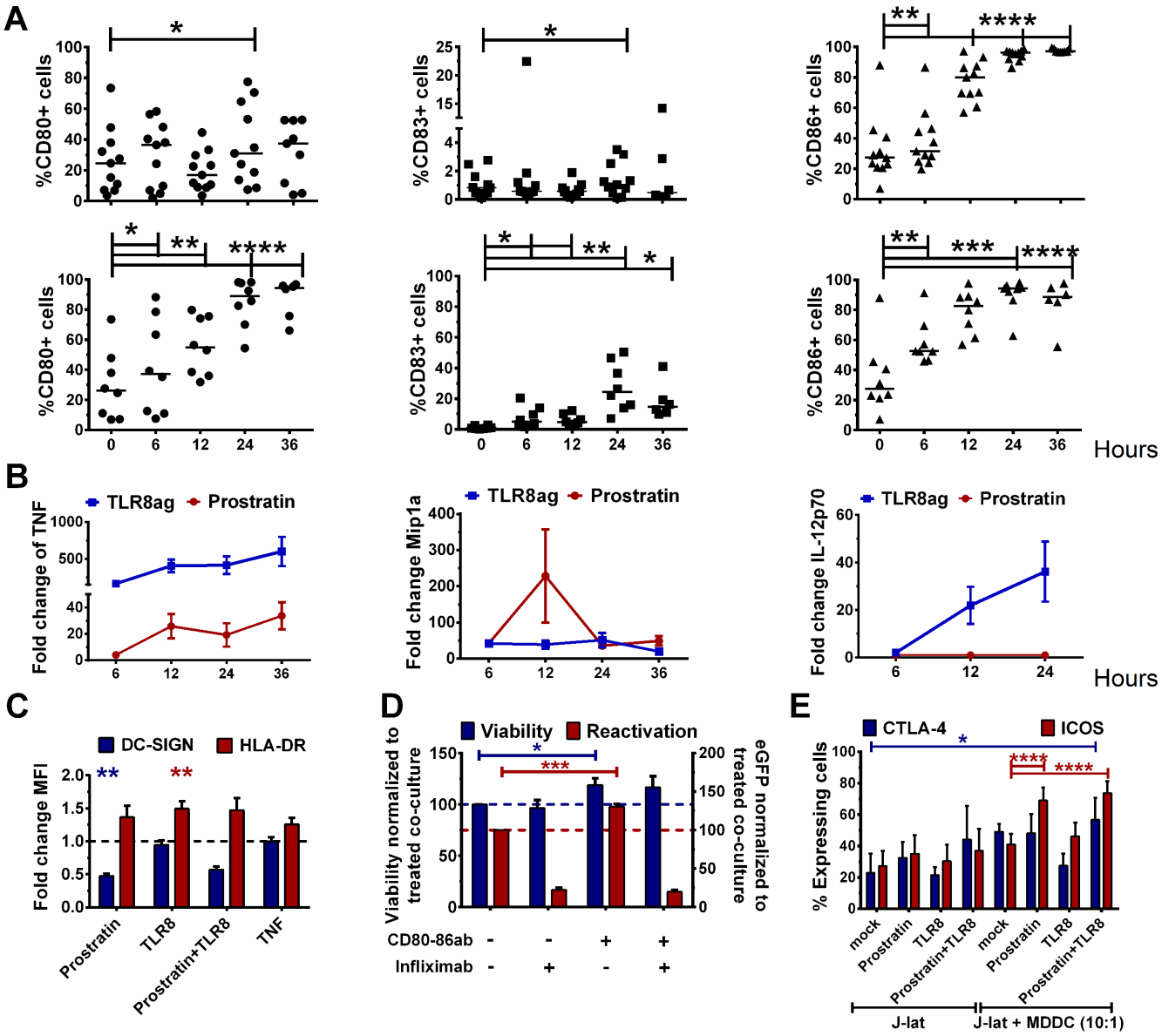
Autologous co-culture of CD4+ T cells (10^5) and MDDCs (10^4) from aviremic patients, in quintuplicate, were stimulated with CD3/CD28/CD2 antibodies at 2.5 µl/ml, Prostratin, TLR8ag or combined as previously, in R-10 medium supplemented with 5 µM of AZT and 50 nM of Efavirenz. Every 2 days, 50 µl of SN per well was collected and pooled per donor and treatment for HIV viral RNA analysis. A) Average peak values from the mock treated controls and the peak values from the treated co-cultures in copies/ml are shown (Mann-Whitney test, **: p=0.0052). B) Peak values of HIV RNA from the individual donors in response to the various LRAs. The dashed line represented the average mock values.

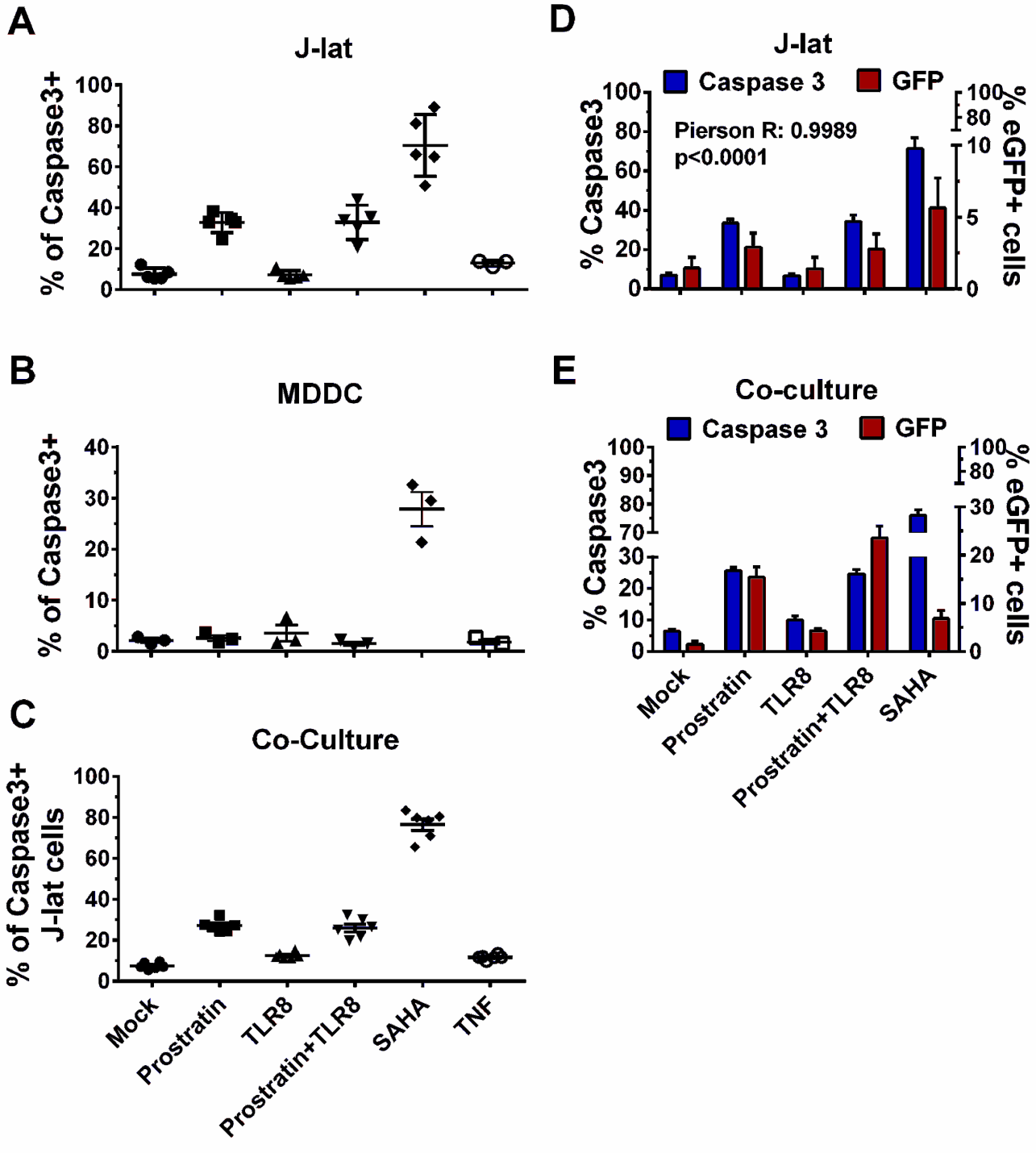


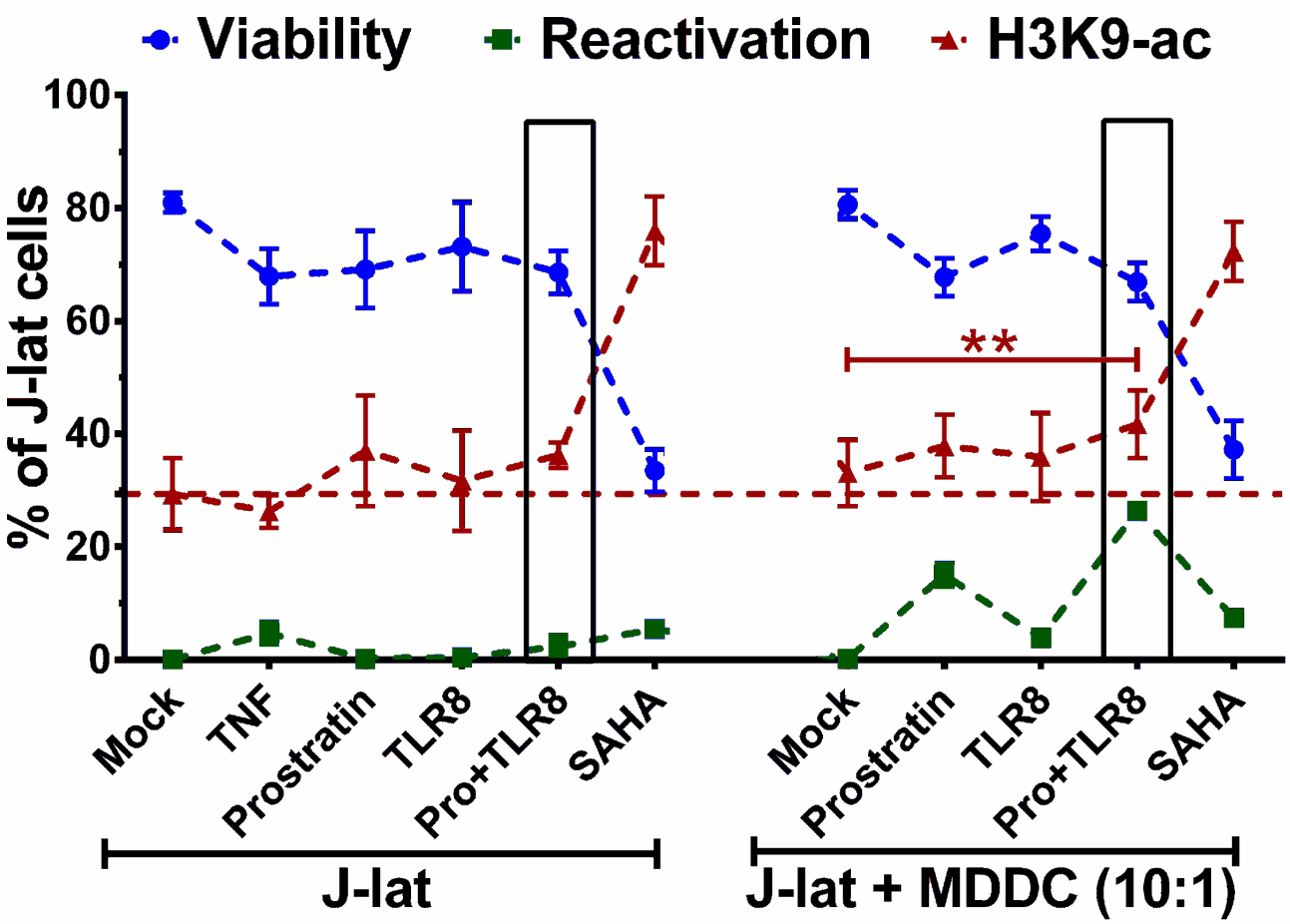


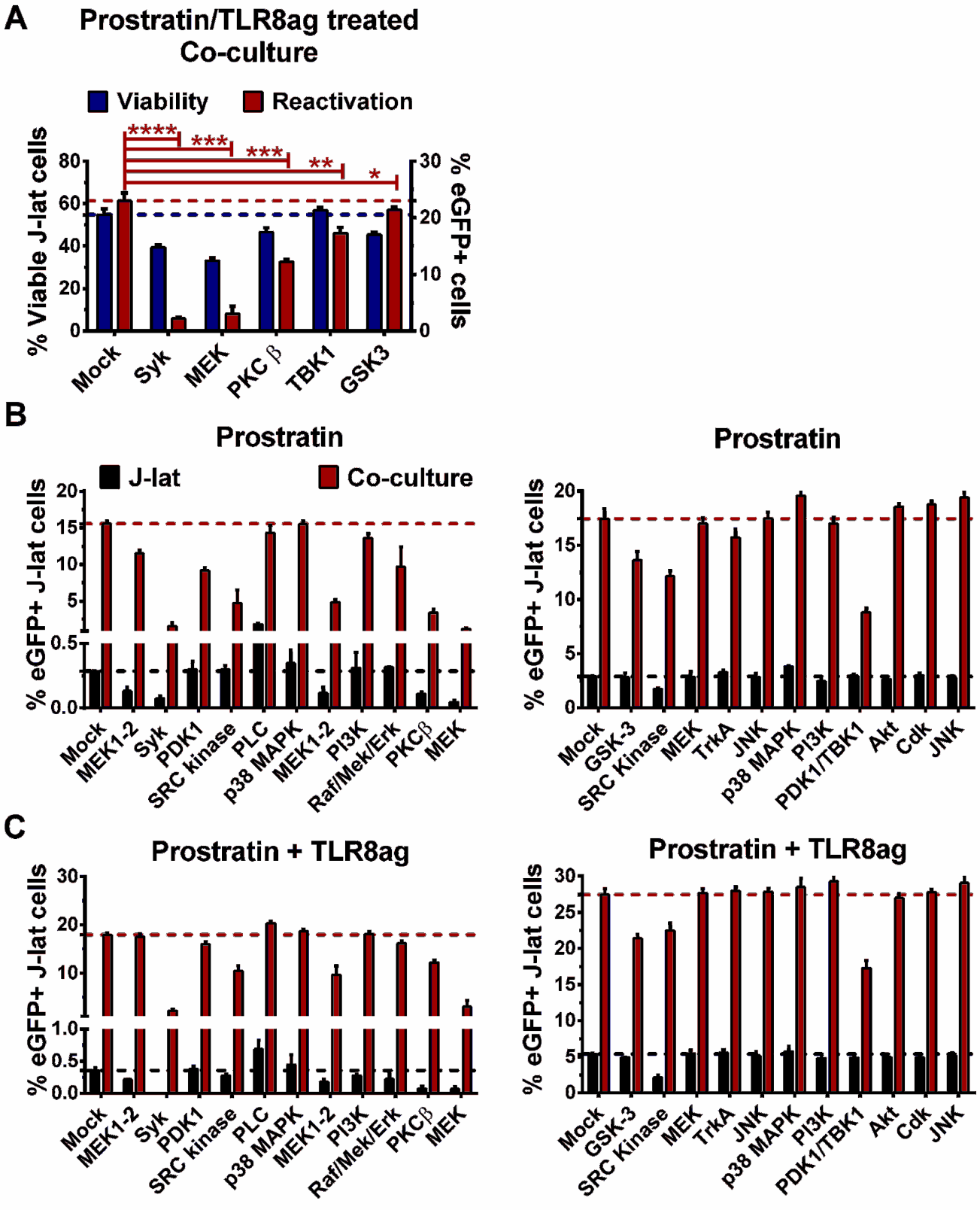












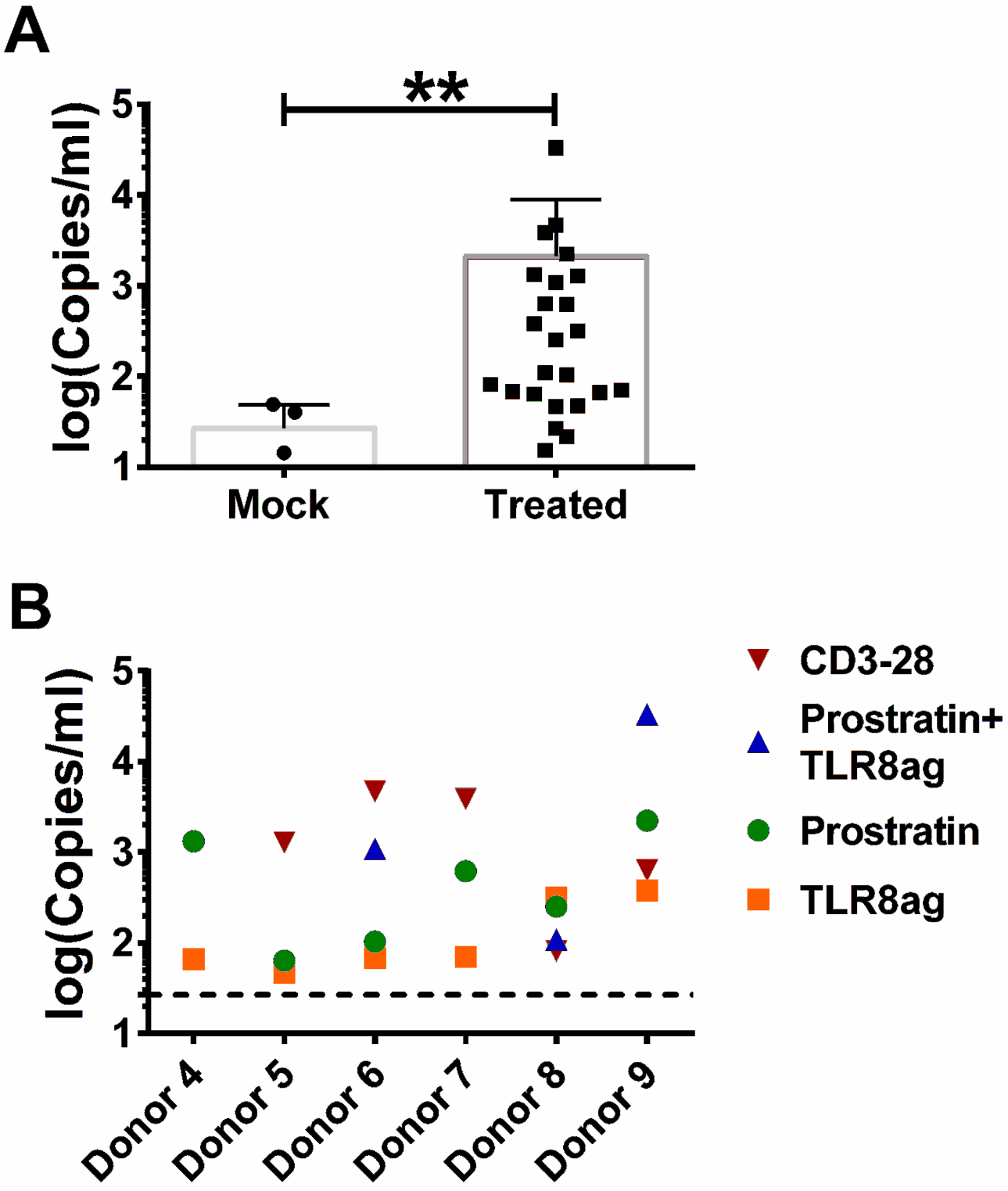


Table 1. Patients characteristics

Patients Number	Age	Gender	MSM	Plasma VL initital [1] (copies/ml)	CD4 initial [1] (cells/µl)	Plasma VL final[2] (copies/ml)	CD4 final[2] (cells/µl)	Duration HIV infection[3] (months)	Time aviremia[4] (months)
4	31	m	No	32'000	265	<20	504	139	71
5	33	f	/	18'800	922	<20	1'140	133	60
6	64	m	Yes	125'500	117	<20	379	207	195
7	47	m	Yes	10'000'000	176	<20	584	44	34
8	47	m	Yes	15'900	137	<20	386	107	71
9	53	m	Yes	962'500	419	<20	1'112	240	122

[1] Plasma viral load and CD4+ T cells counts prior to cART initiation

[2] Plasma viral load and CD4+ T cells counts during the blood sampling

[3] Calculated as the time between the infection and the date of blood sampling

[4] Calculated as the time between the first time point with undetectable viral load and the date of blood sampling